



LEEDS  
BECKETT  
UNIVERSITY

---

Citation:

Vasilca, V and Sadeghpour, A and Rawson, S and Hawke, LE and Baldwin, SA and Wilkinson, T and Bannister, D and Postis, VL and Rappolt, M and Muench, SP and Jeuken, LJC (2018) Spherical-Supported Membranes as Platforms for Screening Against Membrane Protein Targets. *Analytical Biochemistry*, 549. pp. 58-65. ISSN 1096-0309 DOI: <https://doi.org/10.1016/j.ab.2018.03.006>

Link to Leeds Beckett Repository record:

<https://eprints.leedsbeckett.ac.uk/id/eprint/4838/>

Document Version:

Article (Accepted Version)

---

Creative Commons: Attribution-Noncommercial-No Derivative Works 4.0

The aim of the Leeds Beckett Repository is to provide open access to our research, as required by funder policies and permitted by publishers and copyright law.

The Leeds Beckett repository holds a wide range of publications, each of which has been checked for copyright and the relevant embargo period has been applied by the Research Services team.

We operate on a standard take-down policy. If you are the author or publisher of an output and you would like it removed from the repository, please [contact us](#) and we will investigate on a case-by-case basis.

Each thesis in the repository has been cleared where necessary by the author for third party copyright. If you would like a thesis to be removed from the repository or believe there is an issue with copyright, please contact us on [openaccess@leedsbeckett.ac.uk](mailto:openaccess@leedsbeckett.ac.uk) and we will investigate on a case-by-case basis.

# Spherical-Supported Membranes as Platforms for Screening against Membrane Protein Targets

V. Vasilca<sup>a,‡</sup>, A. Sadeghpour<sup>b,c</sup>, S. Rawson<sup>a</sup>, L.E. Hawke<sup>a</sup>, S.A. Baldwin<sup>†,a</sup>, T. Wilkinson<sup>d</sup>,  
D. Bannister,<sup>d</sup> V.L.G. Postis<sup>a,e</sup>, M. Rappolt<sup>b</sup>, S.P. Muench<sup>a</sup> and L.J.C. Jeuken<sup>a,\*</sup>

<sup>a</sup>*The School of Biomedical Sciences and the Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, LS2 9JT, United Kingdom*

<sup>b</sup>*School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, United Kingdom*

<sup>c</sup>*Center for X-ray Analytics, Department of Materials Meet Life, EMPA, 9014 St Gallen, Switzerland.*

<sup>d</sup>*MedImmune, Antibody Discovery and Protein Engineering, Milstein Building, Granta Park, Cambridge, CB21 6GH, United Kingdom*

<sup>e</sup>*Biomedicine Research Group, Faculty of Health and Social Sciences, Leeds Beckett University, Leeds, UK*

<sup>‡</sup> Current address: *The Biochemistry Institute of the Romanian Academy, Bucharest, Romania*

\* Corresponding author: E-mail: [L.J.C.Jeuken@leeds.ac.uk](mailto:L.J.C.Jeuken@leeds.ac.uk)

## Abstract

Screening assays performed against membrane protein targets (e.g. phage display) are hampered by issues arising from protein expression and purification, protein stability in detergent solutions and epitope concealment by detergent micelles. Here, we have studied a fast and simple method to improve screening against membrane proteins: spherical-supported bilayer lipid membranes (“SSBLM”). SSBLMs can be quickly isolated via low-speed centrifugation and redispersed in liquid solutions while presenting the target protein in a native-like lipid environment. To provide proof-of-concept, SSBLMs embedding the polytopic bacterial nucleoside transporter NupC were assembled on 100- and 200 nm silica particles. To test specific binding of antibodies, NupC was tagged with a poly-histidine epitope in one of its central loops between two transmembrane helices. Fluorescent labelling, small angle X-ray scattering (SAXS) and cryo-electron microscopy (cryo-EM) were used to monitor formation of the SSBLMs. Specific binding of an anti-his antibody and a gold-nitrilotriacetic acid (NTA) conjugate probe was confirmed with ELISAs and cryo-EM. SSBLMs for screening could be made with purified and lipid reconstituted NupC, as well as crude bacterial membrane extracts. We conclude that SSBLMs are a promising new means of presenting membrane protein targets for (biomimetic) antibody screening in a native-like lipid environment.

## 34 **Introduction**

35 Encoded by almost one third of archaean, bacterial and eukaryote DNA[1], membrane proteins  
36 represent vital cellular components for all lifeforms. Given their essential roles towards sustaining  
37 life, it is unsurprising that membrane protein pathology accounts for a large number of debilitating  
38 conditions, such as Bartter syndrome, cardiac arrhythmia and hypertension, congenital deafness  
39 and myotonia, cystic fibrosis, epilepsy, osteoporosis and polycystic kidney disease[2, 3]. Their  
40 significant therapeutic importance has led to many of today's pharmaceuticals targeting membrane  
41 proteins[4, 5], with the largest class being the G-protein coupled receptors (GPCRs). However, the  
42 discovery of novel membrane protein binders – including antibody-based medicines that have  
43 emerged throughout the last decade[6] – is not without issue. The high-throughput protocols  
44 employed by the drug discovery industry demand high levels of expression and purity from their  
45 designated screening targets, yet few membrane proteins can be expressed at high level within  
46 their native membranes. Moreover, the general study of membrane proteins is further complicated  
47 by the fact that advanced research techniques (e.g., kinetic and ligand-binding characterisation,  
48 nuclear magnetic resonance (NMR) or X-ray crystallography) cannot always be directly performed  
49 on crude cellular membranes and thus require generous amounts of recombinant protein of high  
50 purity and conformational stability, therefore becoming reliant on identifying optimised  
51 expression platforms, a suitable detergent for the solubilisation and, more often than not,  
52 demanding high-throughput methodologies[7-9].

53 Unfortunately, systems used in the overproduction of membrane protein targets rarely express high  
54 amounts of recombinant protein[10], partly due to differences between the biogenesis pathways of  
55 the host and those of the expression systems and/or the imposed xenobiotic toxicity[8]. Even  
56 following successful expression, membrane proteins are notoriously difficult to purify via standard  
57 techniques such as ion exchange or hydrophobic interaction and poor overall yields can still be  
58 registered after the inclusion of specialised high-affinity chromatography tags[11]. Furthermore,  
59 target denaturation is an ever-present concern after the proteins have been removed from their  
60 native membranes and this is the main reason why detergent solubilisation has been traditionally  
61 used to counter the considerable hydrophobicity and aggregation tendency of membrane proteins  
62 post-purification[7, 9]. While detergent-solubilised proteins facilitate screening with other  
63 biomolecules such as ligands or inhibitors in solution[12], it is commonly desirable to transfer the

64 target proteins into less disruptive environments, since even the mildest detergents can still lead to  
65 the complete inactivation of the solubilised proteins[7]. Moreover, in the context of antibody  
66 binding studies, detergent micelles can also actively block potential epitopes on the chosen  
67 screening targets and can thus have a direct negative impact on the discovery of new antibody-  
68 based pharmaceuticals[12, 13].

69 The main objective of the research presented here was therefore to develop an alternative screening  
70 platform based on spherical-supported bilayer lipid membranes (“SSBLMs”), which can present  
71 membrane protein targets in a native-like lipid environment. SSBLM consist of a solid spherical  
72 core, typically silica, which is coated with lipid membranes. SSBLMs were first developed in the  
73 80s and 90s, are well characterised with spectroscopy and microscopy and their formation has been  
74 well documented (see [14] for a review on SSBLMs). SSBLMs have already been reported for  
75 several membrane proteins, such as the multidrug efflux pump component OprM[15],  
76 bacteriorhodopsin[16] or the redox-driven proton pump cytochrome c oxidase[17]. This prompted  
77 us to explore whether, by refinement of the SSBLM format, this technology can be used in assays  
78 that require or select for specific, high-affinity antibody binding and, eventually, screening assays.  
79 In order to enhance the amount of protein presented in a screening assay, submicron silica particles  
80 were used.

81 In order to provide proof-of-concept for our proposed screening platform, the bacterial nucleoside  
82 transporter NupC was chosen as the membrane protein of interest. Involved in active (secondary)  
83 transport of both purine and pyrimidine nucleosides across bacterial inner membranes (IMs), NupC  
84 is a proton-dependent symporter belonging to the concentrative nucleoside transporter (CNT)  
85 family[18-20]. The protein shares 22-26% amino acid sequence identity with the human  
86 transporters hCNT1-3, which renders it a good model for studying the transport of the therapeutic  
87 nucleoside analogues used in the treatment of life-threatening viral and neoplastic diseases, such  
88 as azidothymidine and gemcitabine[21]. Since antibody-based pharmaceuticals are typically  
89 expected to target epitopes located in the loop regions of transmembrane proteins, a clone of NupC  
90 was engineered to feature a His-tag on one of its central loops, between two transmembrane  
91 helices. This affinity tag allowed for the binding of both anti-His antibodies as well as gold-  
92 conjugated nitrilotriacetic acid (NTA) probes, which greatly aided us in providing our proof-of-  
93 concept.

94 Here, we show that SSBLMs are a suitable platform for screening assays and report on technical  
95 improvements that are required to reduce non-specific binding of antibodies to the SSBLM  
96 particles. Non-specific binding of proteins, including antibodies and biomimetic antibodies, can  
97 occur if silica particles are not completely coated by the lipid membranes, exposing some of the  
98 bare silica surface[22]. Here, we show that including liposomes and bovine serum albumin (BSA),  
99 but not detergents, during the incubation steps with antibodies is a simple and effective strategy to  
100 block non-specific binding. Furthermore, we show that this method can also be applied when using  
101 crude membrane extracts, negating the need to tag and purify membrane proteins in screening  
102 assays.

103

## 104 **Materials and Methods**

### 105 **Materials**

106 All chemicals were purchased from Sigma-Aldrich or Melford unless stated otherwise. His-tagged  
107 NupC detection employed HRP-conjugated mouse IgG<sub>1</sub> anti-His antibodies (R&D Systems,  
108 MAB050H). Lipid, detergent and related materials included 1-palmitoyl-2-oleoyl-*sn*-glycero-3-  
109 phosphocholine (POPC) lipids (Avanti Polar Lipids, 850457),  $\alpha$ -[4-(1,1,3,3-  
110 Tetramethylbutyl)phenyl]-*w*-hydroxy-poly(oxy-1,2-ethanediyl) (Triton X-100) (10% (w/v)  
111 solution) (Anatrace, APX100), Bio-Beads SM-2 adsorbent beads (Bio-Rad, 1523920) and Texas  
112 Red 1,2-Dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (TR-DHPE)  
113 (Thermo Fisher Scientific, T1395MP). Silicon dioxide (SiO<sub>2</sub>) spheres with diameters of 100- and  
114 200 nm were supplied as 10 mg/mL aqueous solutions (nanoComposix, SISN100 and SISN200,  
115 respectively). The peroxidase assay employed a SensoLyte 10-Acetyl-3,7-dihydroxyphenoxazine  
116 (ADHP) peroxidase assay kit (fluorimetric) (AnaSpec, AS-71111). Cryo-EM materials included 5  
117 nm Ni-NTA-Nanogold probes (Nanoprobes, 2082) and lacey carbon film/copper mesh cryo-grids  
118 (Agar Scientific, AGS166).

119

## 120 **NupC cloning**

121 Both an untagged version (pGJL16) as well as a His-tagged construct, of NupC (pLH13), were  
122 used. The plasmid pGJL16 was obtained by cloning the *E. coli nupC* gene into a pTTQ18  
123 vector[23] between EcoRI and HindIII. pTTQ18 features an isopropyl  $\beta$ -D-thiogalactopyranoside  
124 (IPTG)-inducible *tac* promoter[23]. pLH13 was then cloned from pGJL16 through the insertion  
125 of a pentahistidine tag. We previously reported that cloning a His-tag into either the N- or C-  
126 terminus of NupC prevents its expression[24], hence a pentahistidine tag was inserted in the central  
127 cytoplasmic loop between transmembrane (TM) helices 5 and 6, specifically between His230 and  
128 Glu231. The pentahistidine tag, along with the native His230, thus resulted in 6 consecutive  
129 histidines. In pLH13, Cys96 was also mutated to an Ala to reduce potential dimerisation and  
130 aggregation. While the uridine uptake activity of the internally His-tagged NupC construct was  
131 substantially reduced compared to the wild-type variant, its post-purification functionality was  
132 nevertheless retained (unpublished results).

133

## 134 **Purification of His-tagged NupC**

135 The purification of the His-tagged NupC was modified from ref. [24]. pLH13 was transformed  
136 into *E. coli* strain C43 and grown in Lysogeny broth (LB) media supplemented with 100  $\mu$ g/mL  
137 carbenicillin. C43/pLH13 was cultured as 500 mL cultures in 2 L baffled flasks at 37° C with 200  
138 rpm orbital shaking until reaching an OD<sub>600nm</sub> of ~0.6, after which expression was induced with 1  
139 mM IPTG (Generon) for another 4 hours. The cells were then harvested via centrifugation (9000 $\times$ g  
140 for 20 min) and resuspended in 20 mM Tris, 0.5 mM EDTA (pH 7.4) using volumes five to six  
141 times the weight of the harvested cells. Once resuspended, the cells were homogenised using an  
142 Ultra-Turrax cell homogeniser and subsequently lysed via two consecutive runs through a  
143 TS5/40/AB/GA cell disrupter (Constant Systems) at 30 kPsi. The lysed cells were centrifuged at  
144 14,000 $\times$ g for 45 minutes in order to remove cellular debris. The supernatant was ultracentrifuged  
145 at 131,000 $\times$ g for 2 hours to isolate the bacterial membranes. The protein concentration of the  
146 membrane preparation was determined using the bicinchoninic acid (BCA) assay. The membranes  
147 were solubilised in solubilisation buffer (1% (w/v) n-Dodecyl- $\beta$ -D-maltoside (DDM), 50 mM  
148 phosphate buffer, 10% (w/v) glycerol, 150 mM NaCl, 5 mM imidazole and cOmplete™ (EDTA-

149 free) mini protease inhibitor cocktail, pH 7.4) at 4°C for 1 hour at a total membrane protein  
150 concentration of approximately 5 mg/mL. The solubilised membranes were then ultracentrifuged  
151 at 110,000×g for 1 hour, after which the insoluble pellet was discarded. The supernatant was added  
152 to a bed volume of 80 µL of cobalt affinity chromatography resin (Pierce) per 25 mg of total  
153 membrane protein, pre-equilibrated in wash buffer (50 mM phosphate buffer, 10% (w/v) glycerol,  
154 150 mM NaCl, 5 mM imidazole and 0.05% (w/v) DDM, pH 7.4). NupC was bound to the resin  
155 for 16 hours at 4°C with gentle roller mixing. The resin was packed into a disposable filtered  
156 column (Thermo-Pierce Scientific) and washed at 20°C with 10 column volumes of wash buffer.  
157 NupC was eluted in 0.5 mL fractions using elution buffer (50 mM phosphate buffer, 10% (w/v)  
158 glycerol, 150 mM NaCl, 300 mM imidazole and 0.05% (w/v) DDM, pH 7.4) and subsequently  
159 dialysed for another 16 hours at 4°C against dialysis buffer (50 mM MES, 10% (w/v) glycerol,  
160 150 mM NaCl and 0.05% (w/v) DDM, pH 6.8). Finally, the protein samples were concentrated  
161 using a Vivaspin concentrator (Sartorius) with a 10 kDa molecular weight cut off (MWCO). The  
162 concentrated NupC was snap-frozen in liquid nitrogen and stored at -80°C.

163

#### 164 **Liposome preparations**

165 POPC was dissolved in chloroform, distributed into 5 mg aliquots and dried first under a nitrogen  
166 stream, then under vacuum for 2 hours. The desiccated lipid aliquots were stored under a nitrogen  
167 atmosphere at -20° C until used. Liposomes were prepared by first rehydrating the above-  
168 mentioned aliquots in phosphate-buffered saline (PBS), typically at concentrations of 5 mg/mL.  
169 The lipid suspensions were then passed 11 times through a fully assembled Mini-Extruder (Avanti  
170 Polar Lipids), fitted with a polycarbonate track-etched membrane (Whatman) featuring either 100  
171 nm or 200 nm pore sizes, sandwiched between four extruder drain discs (i.e. two on each side of  
172 the membrane). The fluorescent labelling of POPC liposomes was achieved by first dissolving  
173 Texas Red (TR)-modified lipids in a 1:1 (v/v) mixture of chloroform and methanol (0.5 mg/mL)  
174 and adding 100 µL of it to a 5 mg POPC aliquot (i.e. 1% (w/w)) prior to performing the drying  
175 and extrusion steps described above.

176

177 **Inner membrane extract preparation**

178 C43/pGJL16 was cultured as described above and the total membrane fraction was isolated as for  
179 C43/pLH13. Following ultracentrifugation, the membrane pellet (i.e., the total membrane extract)  
180 was resuspended in a 25% (w/w) sucrose Tris/EDTA buffer (20 mM Tris/HCl, 0.5 mM EDTA,  
181 pH 7.5). A 30-55% (w/w) sucrose gradient with centrifugation at 131,000×g for 16 hours was used  
182 to separate the inner membrane (IM) from the outer membrane[25]. The IM fraction was collected  
183 from the gradient and washed with Tris/EDTA buffer via two other 1-hour centrifugations at  
184 131,000×g. The protein concentration of the IM fraction was determined via BCA assay, after  
185 which the IM vesicles were resuspended in Tris/EDTA buffer and stored in 5 mg/mL protein  
186 aliquots at -80°C. For SSBLM formation, the IM vesicles were mixed with POPC liposomes at  
187 various ratios expressed as protein weight of the IM versus dry lipid weight of the POPC vesicles.  
188 The resulting IM/POPC mixture was then snap-frozen in liquid nitrogen and thawed by immersing  
189 the test tube in water at 20° C. This freeze-thaw procedure was repeated three times after which  
190 the IM/POPC mixture was extruded through a 200 nm track-etched membrane as described above.

191

192 **NupC reconstitution**

193 Reconstitution of His-tagged NupC into proteoliposomes was performed following a modified  
194 method of Geertsma *et al*[26]. POPC liposomes (5 mg/mL) in PBS were prepared as detailed above  
195 using 200 nm track-etched membranes. 1 mL of liposomes was titrated with 10% (w/v) Triton X-  
196 100 until  $R_{\text{sat}}$  was reached (as monitored by an increase in  $OD_{540 \text{ nm}}$ ), after which an additional 5  
197  $\mu\text{L}$  of Triton X-100 were added. NupC was mixed in at a protein-lipid ratio of between 1-2.4%  
198 (w/w) and incubated for 15 min at 20° C. Bio-Beads SM-2 (50 mg) were added to remove Triton  
199 X-100 from solution during a 30 min incubation at 20°C under gentle roller mixing. This step was  
200 repeated twice using 60 min and 16 h incubations at 4°C, after which the proteoliposomes were  
201 harvested via ultracentrifugation (100,000×g for 1 hour at 4° C) and resuspended in PBS. Finally,  
202 the proteoliposomes were re-extruded through 200 nm track-etched membrane as described above.

203



204 **SSBLM formation**

205 Silica particles (typically 250  $\mu\text{g}$ ) were vigorously vortexed with liposomes at different lipid-to-  
206 particle ratios (w/w), as indicated in the Results section. Following a 1 hour incubation at 20°C  
207 with gentle roller mixing, the resulting SSBLMs were pelleted via centrifugation (1 min  
208 17,000 $\times g$ ). The supernatants were removed (or transferred into separate tubes, if required), while  
209 the particle pellets were washed by vortexing in identical volumes of deionised water, followed by  
210 a 30 min incubation at 4°C with gentle roller mixing to remove any unbound lipid materials. The  
211 washed SSBLMs were once again harvested by centrifugation and resuspended in PBS prior to  
212 being used or stored at 4°C.

213 Standard procedures were used for SDS-PAGE[27] and Western blot analysis[28]. For Western  
214 blot analysis, SSBLM samples were mixed with SDS-PAGE loading buffer (containing SDS) and  
215 incubated for 1-2 hours at 37<sup>0</sup> degrees to solubilise the membranes and embedded proteins. The  
216 silica particles were removed by a short spin (1 min 17,000 $\times g$ ) and the SDS-PAGE loading buffer  
217 (supernatant) was used to load on the SDS-PAGE.

218

219 **Cryo-electron microscopy (cryo-EM)**

220 100  $\mu\text{L}$  SSBLM samples were created as described above by mixing His-tagged NupC/POPC  
221 proteoliposomes (2% (w/w) protein/lipid ratio) with 200 nm silica particles at a 25% (w/w)  
222 liposome/particle ratio. Protein-free particles were also formed at equivalent concentrations to  
223 serve as negative controls. Following the deionised water wash, the SSBLM samples were pelleted  
224 and resuspended in 100  $\mu\text{L}$  volumes of Ni-NTA-Nanogold probe solution, prepared to a 10:1  
225 probe/protein molar ratio in blocking buffer, consisting of PBS supplemented with 50  $\mu\text{g}/\text{mL}$   
226 POPC vesicles and 1 mg/mL bovine serum albumin (BSA). After a 30 min incubation at 4°C with  
227 gentle roller mixing, the SSBLMs were pelleted and washed twice via vortexing, first in blocking  
228 buffer, then in regular PBS, before being diluted 10 $\times$  further with PBS and applied to the cryo-EM  
229 grids. These were prepared using a FEI Vitrobot Mark IV by first applying 3  $\mu\text{L}$  of sample per  
230 grid (which had previously been glow-discharged for 40 seconds), then blotting off the excess  
231 solution for 2 seconds and finally plunge-freezing the grids in liquid ethane. All of the prepared  
232 cryo-grids were stored in liquid nitrogen prior to being imaged. The grids were imaged at a

233 magnification of 35,000 $\times$  using a FEI Tecnai F20 transmission electron microscope (TEM) fitted  
234 with a Gatan 4K $\times$ 4K charge-coupled device (CCD) camera. All of the images were collected in  
235 “low-dose” mode.

236

### 237 **Small angle X-ray scattering (SAXS)**

238 All small angle X-ray scattering (SAXS) measurements were performed at 20 $^{\circ}$ C. The operated  
239 SAXS camera setup (SAXSpace, Anton Paar, Austria) is described in great detail elsewhere[29].  
240 Briefly, a high-resolution mode was chosen that allowed for the detection of a minimum scattering  
241 vector,  $q_{\min}$ , of 0.04 nm $^{-1}$  ( $q = (4\pi/\lambda) \sin\theta$ , where  $2\theta$  is the scattering angle and  $\lambda$  is the wavelength  
242 of the X-ray beam, namely 0.154 nm). All studied samples were filled into the same vacuum-tight,  
243 reusable 1 mm quartz capillary loading tube, in order to give the exact same scattering volume  
244 each time. A Mythen X-ray detector system (Dectris Ltd., Baden, Switzerland) was used for  
245 recording the 1D scattering patterns. SAXStreat software (Anton Paar, Graz, Austria) was used to  
246 refine the primary beam position. Background due to water and capillary was subtracted using the  
247 SAXSQuant software (Anton Paar, Graz, Austria).

248 Background subtracted SAXS patterns were analysed using an extended core shell model [30].  
249 This model provides the scattering from a spherical core (silica) and six concentric shell structures:  
250 five used to build up the POPC bilayer and one for the space between the silica sphere and the  
251 lipid bilayer (i.e. the intermediate water layer). All electron densities were fixed to literature values  
252 [31, 32], with  $\rho(\text{silica}) = 0.70 \text{ e}/\text{\AA}^3$ ,  $\rho(\text{water}) = 0.33 \text{ e}/\text{\AA}^3$ ,  $\rho(\text{head group}) = 0.45 \text{ e}/\text{\AA}^3$ ,  $\rho(\text{CH}_2) =$   
253  $0.30 \text{ e}/\text{\AA}^3$  and  $\rho(\text{methyl}) = 0.16 \text{ e}/\text{\AA}^3$ . Additionally, the methyl trough and head group thicknesses  
254 were fixed to 0.5 nm and 0.8 nm, respectively. Thus, only three fitting parameters were considered:  
255 (i) the silica radius, (ii) the intermediate water thickness and (iii) the hydrocarbon chain length.  
256 The porosity of the Si-particles was taken into account by determining the form factor contribution  
257 from the nano-pores. For SAXS measurements, both SSBLM and negative control (i.e. bare silica  
258 particle) solutions were created at concentrations of 30 mg/mL in deionised water. In order to get  
259 statistically reliable data and increased signal to noise ratio, we acquired 12 scattering frames each  
260 with 30 minutes exposure time and computed the average curve for the further analysis.

261

## 262 **Enzyme-linked immunosorbent assay (ELISA)**

263 Freshly-made SSBLM particles, resuspended in either regular PBS buffer or blocking buffer (i.e.  
264 PBS supplemented with 50  $\mu\text{g}/\text{mL}$  POPC vesicles and 1  $\text{mg}/\text{mL}$  BSA), were transferred to V-  
265 bottomed 96-well plates and subsequently incubated with 100  $\mu\text{L}$  volumes of HRP-conjugated  
266 anti-His antibodies (diluted 1:5,000 (v/v) in PBS buffer) for 1 hour at 20° C either in PBS or  
267 blocking buffer (10  $\mu\text{g}/\text{mL}$  POPC vesicles and 1  $\text{mg}/\text{mL}$  bovine serum albumin (BSA)) in conical  
268 96-well plates. The particles were pelleted via centrifugation (3,000 $\times$ g, 2 min) and washed twice  
269 in 100  $\mu\text{L}$  volumes of PBS buffer (first with and then without 50  $\mu\text{g}/\text{mL}$  POPC liposomes (10 min  
270 incubations at 20° C). The washed SSBLM pellets were resuspended in 50  $\mu\text{L}$  of PBS buffer and  
271 transferred onto a flat-bottomed 96-well plate. Finally, each test well was supplemented with 50  
272  $\mu\text{L}$  of peroxidase assay working reagent (10-Acetyl-3,7-dihydroxyphenoxazine, ADHP) and  
273 incubated for 30 min at 20°C before the reaction was stopped through the addition of equivalent  
274 volumes of 0.5 M  $\text{H}_2\text{SO}_4$ . Fluorescence was measured at 590/10 nm in a fluorescence plate reader,  
275 with excitation set to 545/10 nm.

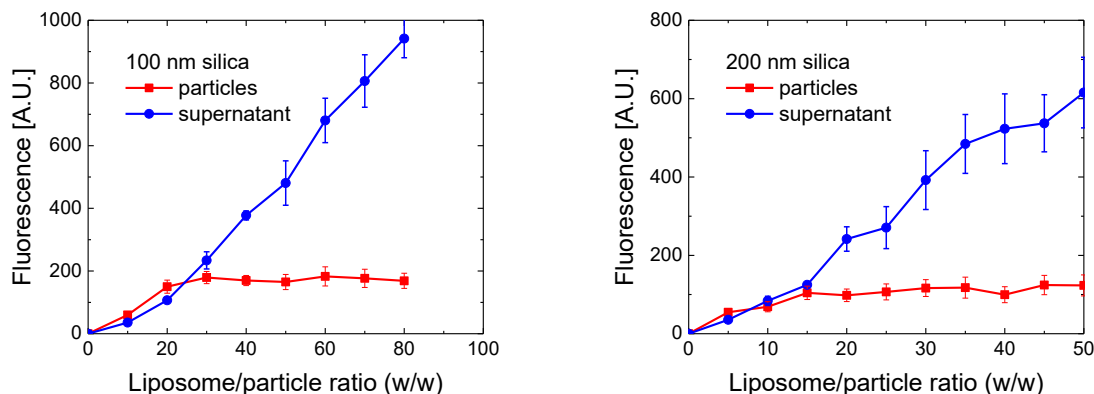
276

277

## 278 **Results**

### 279 **SSBLM formation.**

280 100- and 200 nm silica particles were mixed with fluorescently-labelled POPC vesicles (100 nm)  
281 at different ratios to determine the saturation thresholds resulting in full lipid bilayer coverage of  
282 the particles (Figure 1).



283

284 **Figure 1.** The fluorescence emissions (A.U.) resulting from 100 nm (left) and 200 nm (right) silica particles  
 285 enveloped in fluorescently-labelled POPC SSBLMs (red), as well as from the supernatants obtained after  
 286 pelleting the unwashed particles (blue). The vesicle/particle ratio is given in weight percent. The error bars  
 287 represent the standard error of the mean,  $n = 2$ .

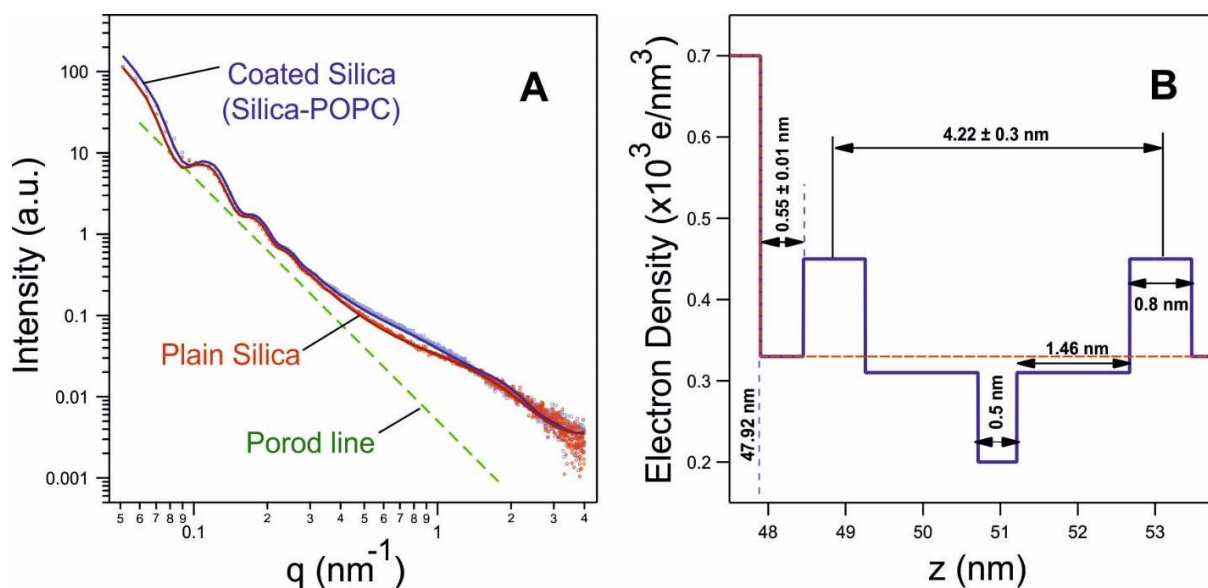
288 These measurements revealed that a minimal vesicle/particle ratio of 30% (w/w) was necessary to  
 289 saturate the 100 nm silica particles, whereas their 200 nm counterparts appeared saturated beyond  
 290 a ratio of 15% (w/w). Such behaviour is expected considering that the surface-to-volume ratio of  
 291 particles scales linearly with their radius. Thus, 200 nm particles will have half the surface area of  
 292 100 nm particles when normalised to the weight of silica. Consequently, about half the amount of  
 293 lipid material is needed to create SSBLMs on 200 nm particles compared to 100 nm particles.

294 While spectrofluorometry proved useful towards indicating whether the POPC lipids were  
 295 adhering to the silica particles to the point of saturation, the results could not discriminate between  
 296 correct SSBLM formation and the simple attachment of lipid material to the available silica  
 297 surface. Therefore, small angle X-ray scattering (SAXS) and cryo-electron microscopy (cryo-EM)  
 298 were used for a more detailed characterisation of the SSBLM particles. EM experiments are  
 299 described below for NupC-embedding SSBLMs.

300 The global fitting analysis of the SAXS data comparing the bare silica to the POPC-coated particles  
 301 confirmed a proper and intact lipid bilayer coating. First, the bare silica particles radius ( $R$ ) was  
 302 determined with a value of  $47.9 \pm 3.5$  nm, consistent with the manufacturer's specifications (Figure  
 303 2A, red). Remarkably, a detailed look into the SAXS profiles of the bare silica particles reveals  
 304 further a deviation from the expected Porod scattering of spheres (Figure 2A, green). Note, the

305 observed additional weak and broad scattering around  $q = 1.7 \text{ nm}^{-1}$  is the form factor contribution  
306 arising from nano-pores ( $R_g = 1.1 \text{ nm}$ ) within the Si-particles.

307 Secondly, the SAXS data from the POPC-coated samples were then fitted with a fixed silica  
308 particle radius of 47.8 nm applying an extended core shell model (see Materials and Methods;  
309 Figure 2A, purple). Not to over-parametrize the model, we kept the number of fitting parameters  
310 as low as possible. Hence, all commonly known electron densities of the modelled SSBLM layers  
311 were set to literature values and further the methyl trough and head group thicknesses were also  
312 fixed to 0.5 nm and 0.8 nm, respectively[31, 32]. This means, only the radial dimensions of the  
313 intermediate water layer thickness and the hydrocarbon chain length were kept as free fitting  
314 parameters (Figure 2B). The fitting results are shown in panel A as solid lines and are in excellent  
315 agreement with the recorded data points. The scattering contribution of the POPC bilayer coating  
316 is most dominant in the range of  $0.4 < q < 1.1 \text{ nm}^{-1}$ , in which its form factor scattering contribution  
317 is recorded. Note, this  $q$ -range is well separated from the highest scattering contribution arising  
318 from the silica nano-pores, and hence, the evaluation of bilayer structure is unproblematic. In  
319 conclusion, the SAXS data analysis supports the proper and intact formation of the SSBLMs  
320 displaying a bilayer phosphate-to-phosphate distance of the supported lipid bilayers to be  $4.2 \pm 0.3$   
321 nm at 20°C, which within errors agrees with the previously reported value of 3.9 nm by Kučerka  
322 *et al*[33]. The fit also included the water layer for which a thickness of  $0.55 \pm 0.01 \text{ nm}$  was  
323 determined, thinner than the 1.7 nm determined by Bayerl *et al.* by NMR for SSBLMs [34].  
324 However, others have reported a large spread in the thickness of the water layer on planer  
325 substrates (e.g., compare ref [35] with [36]), some with a thickness between 0.2 and 0.8 nm [36].

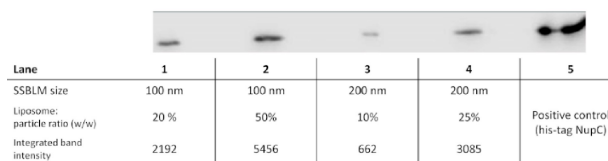


326  
 327 **Figure 2.** SAXS analysis of bare and POPC-coated silica particles. (A) The scattering profiles resulting  
 328 from stock R ~ 48 nm silica particles (red) and POPC SSBLMs (purple) alongside their corresponding fit  
 329 functions (solid lines). The green dashed line shows the linear decay based on Porod's law for scattering  
 330 from ideal spheres. (B) Refined electron density profile of our applied SSBLM model.

331

332 **Protein incorporation into SSBLMs.**

333 In order to embed membrane proteins into SSBLMs, fresh samples were formed using NupC-  
 334 containing proteoliposomes. His-tagged NupC was first reconstituted into POPC vesicles at a 2.4%  
 335 (w/w) protein/lipid ratio and the resulting proteoliposomes were subsequently used in the  
 336 formation of both 100- and 200 nm NupC-embedding SSBLMs, as described under Materials and  
 337 Methods. The successful embedding of NupC into the SSBLM was initially confirmed via SDS-  
 338 PAGE and western blotting, using an HRP-conjugated anti-His antibody (Figure 3). The  
 339 appearance of the bands confirmed that NupC was indeed present within the SSBLMs, while the  
 340 intensity of the NupC bands from the 100 nm SSBLM samples was significantly more intensive  
 341 than that of the 200 nm SSBLM, as expected, given the proportionate increase in surface area (both  
 342 SSBLM sample sizes used an identical amount of silica particles, so the 100 nm particles have  
 343 double the surface area of the 200 nm).



344

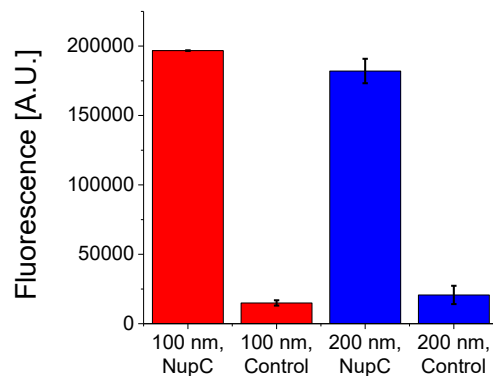
345 **Figure 3.** Western blot of 100- and 200 nm POPC SSBLMs embedding His-tagged NupC. SSBLMs were  
 346 prepared as described in the text at different liposome/silica particle ratios as indicated in the Figure.  
 347 Identical weights of silica are used in the Western blot. Purified His-tagged NupC was used as positive  
 348 control in lane 5 and quantitative band intensities are given.

349

### 350 **Specific binding of antibodies and gold-conjugated Ni-NTA probes**

351 The suitability of SSBLMs for selective screening was tested through a peroxidase ELISA assay.  
 352 Protein-free SSBLM particles were created for negative control purposes. It should be noted that,  
 353 in order to preserve the structure of the SSBLMs, the antibody incubation step was performed in  
 354 the absence of detergents typical of traditional ELISAs (i.e. Tween-20). Initial results revealed  
 355 high background signals, due to non-specific binding of the antibody to SSBLM, presumable as a  
 356 consequence of defects in the membrane coating, exposing the bare silica[22]. However, non-  
 357 specific binding of antibodies could be blocked via the addition of 50 µg/mL POPC liposomes and  
 358 1 mg/mL BSA during the antibody incubation step. The 50 µg/mL POPC vesicles were added to  
 359 all of the subsequent washing steps to repair any defects in the membrane layer that might arise  
 360 during the assay[22]. The results obtained using this optimised protocol minimized non-specific  
 361 antibody binding and confirmed that SSBLM can be used to bind (and hence screen) antibodies to  
 362 membrane proteins in native-like lipid environment (Figure 4).

363



364

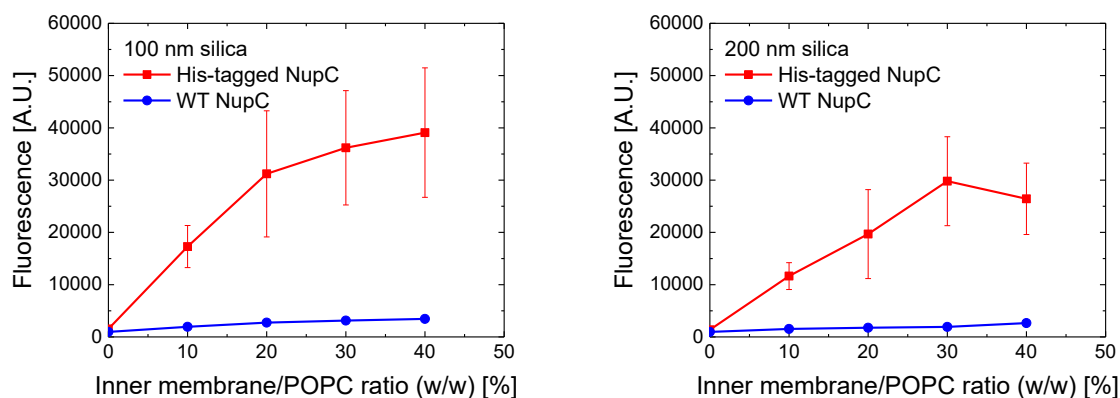
365 **Figure 4.** ELISA results (fluorescence signals emitted) from 100 nm (red) and 200 nm (blue) silica particles  
 366 enveloped in protein-free POPC SSBLMs (control) or SSBLMs embedding His-tagged NupC (NupC).  
 367 Control and NupC SSBLMs were treated identically. The error bars represent the standard error of the  
 368 mean, n = 2.

369 Using our optimised peroxidase assay protocol, we also tested whether SSBLMs could be formed  
 370 directly from total (i.e. “crude”) IM extracts overexpressing our protein of interest, since such an  
 371 approach would prove highly beneficial towards assaying membrane protein targets that are  
 372 difficult to purify or reconstitute into lipid vesicles. We have previously shown that to deposit  
 373 solid-supported membranes on planar glass or silica surfaces using crude bacterial membranes,  
 374 such extracts have to be first mixed with liposomes (e.g. POPC liposomes) to reduce protein  
 375 content in the membranes[37]. POPC vesicles were thus mixed via the freeze-thaw method with  
 376 *E. coli* IM extracts overexpressing His-tagged NupC at various ratios. Membrane extracts  
 377 overexpressing the untagged/wild-type construct of NupC were used as negative controls. The  
 378 results (Figure 5) confirmed that SSBLMs can also be used as a screening platform when crude  
 379 IM extracts, containing high protein-to-lipid ratios, are used. The optimum ratio of bacterial IM  
 380 extracts to POPC liposomes lies between 20-40% (w/w), in line with previous findings on planar  
 381 surfaces[37], suggesting this optimal ratio is independent on the target proteins that is studied.  
 382 Figure 5 shows larger values for the standard error of mean when compared to Figure 4. Comparing  
 383 individual experiments shows that this is due to varying amounts of target proteins incorporated  
 384 from the crude membrane extract into the SSBLM, as errors are similar to those in Figure 4 when  
 385 ELISAs are performed on the same SSBLM batch. We propose that this due to the need to mix  
 386 crude membrane extracts with POPC liposomes, which might result in slight variations in



387 incorporation of membrane proteins into the SSBLMs, even when fixed ratios of POPC versus  
388 crude membrane extracts are used.

389



390

391 **Figure 5.** ELISA results (fluorescence signals emitted) from 100 nm (left) and 200 nm (right) silica particles  
392 enveloped in untagged NupC-expressing total membrane extracts (blue), as well as IM extracts  
393 overexpressing His-tagged NupC (red). Both IM extracts were mixed with POPC vesicles at different ratios,  
394 as indicated. The error bars represent the standard error of the mean, n = 3.

395 Although the western blot and ELISA experiments confirmed the presence of His-tagged NupC  
396 embedded onto the silica particles, these two methods do not confirm that the lipid bilayers were  
397 correctly forming around the particles as compared to, for instance, intact vesicles adsorbing to the  
398 surface of the particles. Indeed, the negative effects of membrane proteins on the formation of  
399 planar solid supported membranes have previously been documented[38]. We note here, however,  
400 that even should the vesicles be (partly) unfused on the spherical silica particles, the system would  
401 still form a suitable screening platform as indicated by the ELISA results. Nevertheless, in order  
402 to further confirm the correct formation of the SSBLMs, as well as to provide a second method to  
403 show specific binding to embedded proteins, we also imaged the SSBLM particles via cryo-EM.

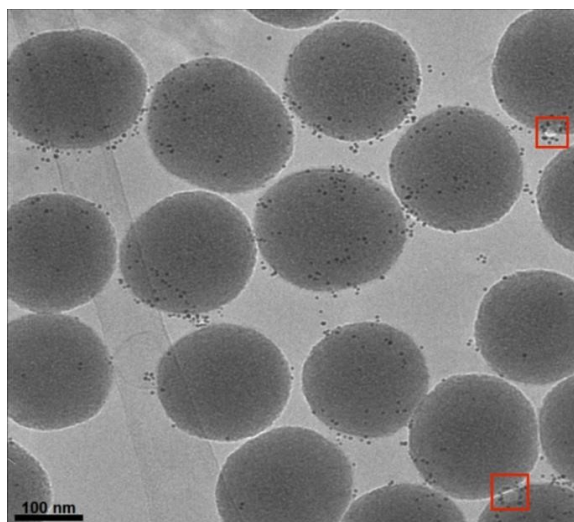
404 EM samples were prepared using His-tagged NupC/POPC proteoliposomes (and protein-free  
405 POPC liposomes for the equivalent negative controls) and subsequently incubated with Ni-NTA-  
406 Nanogold probes so as to monitor the distribution of His-tagged NupC (Figure 6). By rapidly  
407 freezing the SSBLMs in vitreous ice, the lipid membrane structure is preserved as opposed to  
408 negative staining, which can flatten the specimens being studied. In order to further preserve the

409 quality of the images, a “low-dose” exposure procedure was used such that the electron radiation  
410 damage could be minimised. Under these conditions, the discrete lipid membrane components of  
411 the SSBLMs could not be directly observed (in contrast to previous studies[15, 39]), but the bound  
412 Ni-NTA-Nanogold particles clearly indicated the location of the embedded proteins on the surface  
413 of the SSBLMs, highlighted by the multitude of representative black “dots”. The images show that  
414 Ni-NTA binding was indeed specific to his-tagged NupC within the SSBLMs and that membrane  
415 envelope the silica particles to form a SSBLM (i.e., not adsorbed as intact proteoliposomes). We  
416 note, however, that for less than 1 in 5 SSBLM particles, unfused vesicles were also visible. Two  
417 examples of unfused vesicles are indicated by a red box in Figure 6, although others examples .

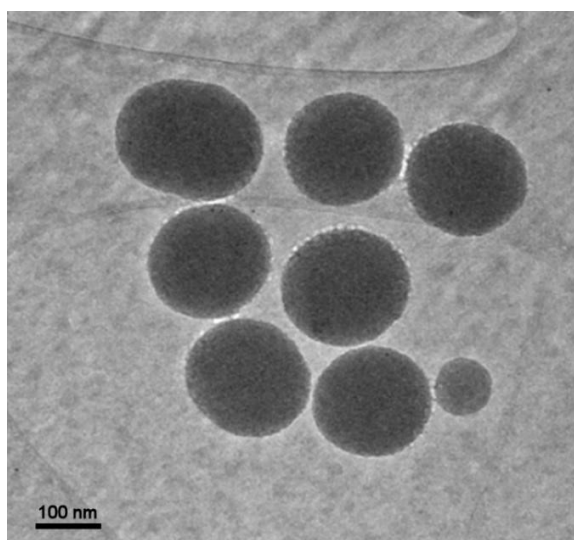
418 Analysing a number of EM images, a distribution of 5 to 60 Ni-NTA-Nanogold were observed per  
419 silica particle, with an average of 30 Ni-NTA-Nanogold/silica particle (S.D. = 18). Taking the  
420 diameter of the silica particles as 200 nm, the molecular weight of NupC to be 44 kDa, the surface  
421 area of POPC as  $67 \text{ \AA}^2$  (MW 760 Da), it can be estimated that 130 NupC proteins are present for  
422 each silica particle (a 2% (w/w) ratio of NupC to lipid was used to prepare the proteoliposomes).  
423 If NupC adopts a random orientation on the silica particles, half of them will have the His-tag  
424 facing outwards for the Ni-NTA-Nanogold to bind, i.e. 65 per silica. The lower average number  
425 of Ni-NTA-Nanogold that are observed in the EM images could be due to incomplete binding of  
426 the Ni-NTA-Nanogold to the exposed his-tags or loss of NupC during the reconstitution into  
427 proteoliposomes.

428

429



430



431 **Figure 6.** Cryo-EM images of 200 nm silica particles coated with POPC SSBLMs with (top) and without  
432 (bottom) embedded His-tagged NupC after incubation with 5 nm gold-conjugated Ni-NTA probes. The two  
433 red boxes in the top figure indicate two examples of unfused vesicles.

434

## 435 **Discussion**

436 A particular screening method that finds increasing use in both the pharmaceutical and  
437 biotechnological fields is that based on phage display. In principle, phage display screening can  
438 be performed using detergent-solubilised membrane protein targets. However, detergent-based  
439 screening methods come with their own drawbacks, including target denaturation over long

440 periods of storage or the inability to solubilise certain membrane protein classes due to monomer  
441 packing defects resulting in their aggregation and, ultimately, inactivation following  
442 purification[40]. A final problem with phage display screening against membrane protein targets  
443 is the immobilisation strategy. Globular proteins are typically adsorbed onto polymeric or  
444 streptavidin coated surfaces. However, detergent solubilisation of membrane proteins and the  
445 aforementioned problems with tagging can impede these strategies. Several alternative strategies  
446 have been described, such as whole cell panning[41] or embedding the proteins into nanodiscs[42].  
447 Whole cells provide a very complex environment for screening while nanodiscs still require the  
448 membrane proteins to be purified to a high yield and purity.

449 By combining the attractive properties of both submicron materials and model membranes, our  
450 proposed SSBLM particles aim to become an improved antigen presentation method available to  
451 membrane protein researchers. The successful embedding of NupC within the SSBLM format on  
452 both 100 and 200 nm silica particles, along with confirming the accessibility of the embedded  
453 proteins towards high-affinity antibody binding, both suggest that the SSBLMs could constitute a  
454 promising new means of studying membrane proteins in the future. SSBLMs represent a versatile  
455 model system that not only mimics the original cellular lipid environment, but also elegantly  
456 circumvents the numerous disadvantages offered by traditional detergent solubilisation methods.  
457 Although the ‘shelf-life’ stability of the SSBLM was not studied here, membrane proteins have  
458 previously been determined to be stable of weeks in silica-supported membrane systems[43].  
459 Therefore, we believe that the platform could ultimately serve as an enhanced screening support  
460 for the discovery of novel antibody binders in an industrial setting, using high-throughput  
461 technologies, just as it has already been considered for the role of delivering therapeutic payloads  
462 to membrane protein targets via SSBLM-based nanovectors[39].

463 We note here that suitable liposome/particle ratios must be met in order to avoid partially covered  
464 substrates, which can result in non-specific binding. Just as importantly, the blocking of non-  
465 specific binding sites and the washing of unbound materials must both be carefully considered in  
466 order to reduce the chances of obtaining false positive results. To this end, the format would greatly  
467 benefit from a faster washing procedure and one promising alternative would be to assemble the  
468 SSBLMs on iron oxide-core, silica-shell particles, so as to facilitate their magnetic separation from  
469 solution and thus eliminate the platform’s reliance on the more time-consuming centrifugation-

470 based pelleting. Superparamagnetic ferrite particles have already been covered with lipid bilayers  
471 in the past[39] and an added benefit of other such improvements would be the possibility for further  
472 automation offered by an industrial setting, which would ultimately allow the SSBLMs to be used  
473 in high-throughput scenarios as well.

474 Other improvements could be considered for the use of SSBLMs in screening assays. Some  
475 approaches enable the oriented reconstitution of appropriately-tagged membrane proteins (e.g.  
476 His-tagged proteolipid bilayers deposited onto Ni-NTA-treated surfaces[44]), while others are  
477 better suited at preserving the functionality of the target proteins post-purification (e.g. SSBLMs  
478 for electrophysiology[45, 46] or electrochemistry[47-53]). Any contact with a solid support can  
479 potentially affect protein fluidity across the model membrane and, consequently, prevent the  
480 uniform distribution of the designated antigen throughout the chosen screening format. Several  
481 alternatives to the conventional method of solid supported membrane formation via  
482 proteoliposomal deposition have been trialled in an attempt at bypassing the problems caused by  
483 protein immobility or improper membrane solubilisation using detergents[7], such as the self-  
484 insertion of purified membrane proteins into an already-formed solid supported membrane[15] or  
485 the formation of a polyethylene glycol (PEG)-supported bilayer[54-56].

## 486 **Conclusion**

487 In conclusion, we have demonstrated that SSBLMs represent a promising platform for screening  
488 assays, where membrane protein targets are displayed embedded within a native-like lipid  
489 environment. We have also demonstrated that SSBLMs can be quickly and easily formed using  
490 purified proteins reconstituted into liposomes, as well as by directly employing crude membrane  
491 extracts. Here, the potential suitability of the SSBLM platform towards high-affinity antibody  
492 binding was established using ELISAs and cryo-EM imaging, where the former technique showed  
493 that non-specific binding can be minimised through suitable assay modifications. We are now  
494 investigating whether the SSBLM can be applied in phage display screening.

## 495 **Acknowledgements**

496 The work presented herein was funded through a BBSRC CASE PhD studentship (BB/J011347/1).  
497 During this work SR was a Wellcome Trust supported PhD student (009752/Z/12/Z).

498 **References**

- 499 [1] E. Wallin, G. von Heijne, Genome-wide analysis of integral membrane proteins from  
500 eubacterial, archaean, and eukaryotic organisms, *Protein Sci.*, 7 (1998) 1029-1038.
- 501 [2] A. Aperia, Membrane transport proteins in health and disease, *J. Intern. Med.*, 261 (2007) 2-4.
- 502 [3] C.A. Hubner, T.J. Jentsch, Ion channel diseases, *Hum. Mol. Genet.*, 11 (2002) 2435-2445.
- 503 [4] A.L. Hopkins, C.R. Groom, The druggable genome, *Nat. Rev. Drug Discov.*, 1 (2002) 727-  
504 730.
- 505 [5] J.P. Overington, B. Al-Lazikani, A.L. Hopkins, How many drug targets are there?, *Nat. Rev.*  
506 *Drug Discov.*, 5 (2006) 993-996.
- 507 [6] K. Imai, A. Takaoka, Comparing antibody and small-molecule therapies for cancer, *Nat. Rev.*  
508 *Cancer*, 6 (2006) 714-727.
- 509 [7] A.M. Seddon, P. Curnow, P.J. Booth, Membrane proteins, lipids and detergents: not just a soap  
510 opera, *Biochim. Biophys. Acta*, 1666 (2004) 105-117.
- 511 [8] F. Mancia, J. Love, High-throughput expression and purification of membrane proteins, *J.*  
512 *Struct. Biol.*, 172 (2010) 85-93.
- 513 [9] R.M. Bill, P.J. Henderson, S. Iwata, E.R. Kunji, H. Michel, R. Neutze, S. Newstead, B.  
514 Poolman, C.G. Tate, H. Vogel, Overcoming barriers to membrane protein structure determination,  
515 *Nat. Biotechnol.*, 29 (2011) 335-340.
- 516 [10] M. Rahman, F. Ismat, M.J. McPherson, S.A. Baldwin, Topology-informed strategies for the  
517 overexpression and purification of membrane proteins, *Mol. Membr. Biol.*, 24 (2007) 407-418.
- 518 [11] A.K. Mohanty, M.C. Wiener, Membrane protein expression and production: effects of  
519 polyhistidine tag length and position, *Protein Expr. Purif.*, 33 (2004) 311-325.
- 520 [12] M. le Maire, P. Champeil, J.V. Moller, Interaction of membrane proteins and lipids with  
521 solubilizing detergents, *Biochim. Biophys. Acta*, 1508 (2000) 86-111.
- 522 [13] E.R. Kunji, M. Harding, P.J. Butler, P. Akamine, Determination of the molecular mass and  
523 dimensions of membrane proteins by size exclusion chromatography, *Methods*, 46 (2008) 62-72.
- 524 [14] A.L. Troutier, C. Ladaviere, An overview of lipid membrane supported by colloidal particles,  
525 *Adv. Colloid Interface Sci.*, 133 (2007) 1-21.
- 526 [15] S. Trepout, S. Mornet, H. Benabdelhak, A. Ducruix, A.R. Brisson, O. Lambert, Membrane  
527 protein selectively oriented on solid support and reconstituted into a lipid membrane, *Langmuir*,  
528 23 (2007) 2647-2654.

- 529 [16] R.W. Davis, A. Flores, T.A. Barrick, J.M. Cox, S.M. Brozik, G.P. Lopez, J.A. Brozik,  
530 Nanoporous microbead supported bilayers: stability, physical characterization, and incorporation  
531 of functional transmembrane proteins, *Langmuir*, 23 (2007) 3864-3872.
- 532 [17] G. Nordlund, J.B. Sing Ng, L. Bergstrom, P. Brzezinski, A membrane-reconstituted  
533 multisubunit functional proton pump on mesoporous silica particles, *ACS Nano*, 3 (2009) 2639-  
534 2646.
- 535 [18] S.K. Loewen, S.Y. Yao, M.D. Slugoski, N.N. Mohabir, R.J. Turner, J.R. Mackey, J.H.  
536 Weiner, M.P. Gallagher, P.J. Henderson, S.A. Baldwin, C.E. Cass, J.D. Young, Transport of  
537 physiological nucleosides and anti-viral and anti-neoplastic nucleoside drugs by recombinant  
538 *Escherichia coli* nucleoside-H<sup>+</sup> cotransporter (NupC) produced in *Xenopus laevis* oocytes, *Mol.*  
539 *Membr. Biol.*, 21 (2004) 1-10.
- 540 [19] S.G. Patching, S.A. Baldwin, A.D. Baldwin, J.D. Young, M.P. Gallagher, P.J. Henderson,  
541 R.B. Herbert, The nucleoside transport proteins, NupC and NupG, from *Escherichia coli*: specific  
542 structural motifs necessary for the binding of ligands, *Org. Biomol. Chem.*, 3 (2005) 462-470.
- 543 [20] J.E. Craig, Y. Zhang, M.P. Gallagher, Cloning of the nupC gene of *Escherichia coli* encoding  
544 a nucleoside transport system, and identification of an adjacent insertion element, IS 186, *Mol.*  
545 *Microbiol.*, 11 (1994) 1159-1168.
- 546 [21] J.A. Thorn, S.M. Jarvis, Adenosine transporters, *Gen. Pharmacol.*, 27 (1996) 613-620.
- 547 [22] J. Drazenovic, S. Ahmed, N.-M. Tuzinkiewicz, S.L. Wunder, Lipid exchange and transfer on  
548 nanoparticle supported lipid bilayers: effect of defects, ionic strength, and size, *Langmuir*, 31  
549 (2015) 721-731.
- 550 [23] M.J.R. Stark, Multicopy expression vectors carrying the lac repressor gene for regulated high-  
551 level expression of genes in *Escherichia coli*, *Gene*, 51 (1987) 255-267.
- 552 [24] C. Ma, Z.Y. Hao, G. Huysmans, A. Lesiuk, P. Bullough, Y.Y. Wang, M. Bartlam, S.E.  
553 Phillips, J.D. Young, A. Goldman, S.A. Baldwin, V.L.G. Postis, A versatile strategy for production  
554 of membrane proteins with diverse topologies: application to investigation of bacterial homologues  
555 of human divalent metal ion and nucleoside transporters, *Plos One*, 10 (2015) ARTN e0143010.
- 556 [25] C.A. Schnaitman, Protein composition of cell wall and cytoplasmic membrane of *Escherichia*  
557 *coli*, *J Bacteriol*, 104 (1970) 890-901.
- 558 [26] E.R. Geertsma, N.A.B. Nik Mahmood, G.K. Schuurman-Wolters, B. Poolman, Membrane  
559 reconstitution of ABC transporters and assays of translocator function, *Nat. Protoc.*, 3 (2008) 256-  
560 266.
- 561 [27] U.K. Laemmli, Cleavage of structural proteins during assembly of head of bacteriophage T4,  
562 *Nature*, 227 (1970) 680-685.

- 563 [28] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide  
564 gels to nitrocellulose sheets - procedures and some applications, Proc. Natl. Acad. Sci. U. S. A.,  
565 76 (1979) 4350-4354.
- 566 [29] Y. Patil-Sen, A. Sadeghpour, M. Rappolt, C.V. Kulkarni, Facile preparation of internally self-  
567 assembled lipid particles stabilized by carbon nanotubes, Jove-J. Vis. Exp., (2016) Artn e53489.
- 568 [30] A. Guinier, G. Fournet, Small-angle scattering of X-Rays, John Wiley and Sons, New York,  
569 1955.
- 570 [31] P.E. Harper, D.A. Mannoock, R.N.A.H. Lewis, R.N. McElhaney, S.M. Gruner, X-ray  
571 diffraction structures of some phosphatidylethanolamine lamellar and inverted hexagonal phases  
572 (vol 81, pg 2693, 2001), Biophys. J., 102 (2012) 1236-1236.
- 573 [32] P.E. Harper, D.A. Mannoock, R.N.A.H. Lewis, R.N. McElhaney, S.M. Gruner, X-ray  
574 diffraction structures of some phosphatidylethanolamine lamellar and inverted hexagonal phases,  
575 Biophys. J., 81 (2001) 2693-2706.
- 576 [33] N. Kucerka, M.P. Nieh, J. Katsaras, Fluid phase lipid areas and bilayer thicknesses of  
577 commonly used phosphatidylcholines as a function of temperature, BBA - Biomembranes, 1808  
578 (2011) 2761-2771.
- 579 [34] T.M. Bayerl, M. Bloom, Physical properties of single phospholipid bilayers adsorbed to micro  
580 glass beads. A new vesicular model system studied by <sup>2</sup>H-nuclear magnetic resonance, Biophys.  
581 J., 58 (1990) 357-362.
- 582 [35] S.J. Johnson, T.M. Bayerl, D.C. McDermott, G.W. Adam, A.R. Rennie, R.K. Thomas, E.  
583 Sackmann, Structure of an adsorbed dimyristoylphosphatidylcholine bilayer measured with  
584 specular reflection of neutrons, Biophys. J., 59 (1991) 289-294.
- 585 [36] B.W. Koenig, S. Krueger, W.J. Orts, C.F. Majkrzak, N.F. Berk, J.V. Silverton, K. Gawrisch,  
586 Neutron reflectivity and atomic force microscopy studies of a lipid bilayer in water adsorbed to  
587 the surface of a silicon single crystal, Langmuir, 12 (1996) 1343-1350.
- 588 [37] C.E. Dodd, B.R. Johnson, L.J. Jeuken, T.D. Bugg, R.J. Bushby, S.D. Evans, Native *E. coli*  
589 inner membrane incorporation in solid-supported lipid bilayer membranes, Biointerphases, 3  
590 (2008) FA59.
- 591 [38] A. Granéli, J. Rydström, B. Kasemo, F. Höök, Formation of supported lipid bilayer  
592 membranes on SiO<sub>2</sub> from proteoliposomes containing transmembrane proteins, Langmuir, 19  
593 (2003) 842-850.
- 594 [39] S. Mornet, O. Lambert, E. Duguet, A. Brisson, The formation of supported lipid bilayers on  
595 silica nanoparticles revealed by cryoelectron microscopy, Nano Lett., 5 (2005) 281-285.
- 596 [40] R.M. Garavito, S. Ferguson-Miller, Detergents as tools in membrane biochemistry, J. Biol.  
597 Chem., 276 (2001) 32403-32406.



- 598 [41] M.L. Jones, M.A. Alfaleh, S. Kumble, S. Zhang, G.W. Osborne, M. Yeh, N. Arora, J.J.C.  
599 Hou, C.B. Howard, D.Y. Chin, S.M. Mahler, Targeting membrane proteins for antibody discovery  
600 using phage display, *Sci. Rep.*, 6 (2016) Artn 26240.
- 601 [42] P.K. Dominik, M.T. Borowska, O. Dalmas, S.S. Kim, E. Perozo, R.J. Keenan, A.A.  
602 Kossiakoff, Conformational chaperones for structural studies of membrane proteins using  
603 antibody phage display with nanodiscs, *Structure*, 24 (2016) 300-309.
- 604 [43] G. Puu, E. Artursson, I. Gustafson, M. Lundström, J. Jass, Distribution and stability of  
605 membrane proteins in lipid membranes on solid supports, *Biosensors and Bioelectronics*, 15  
606 (2000) 31-41.
- 607 [44] F. Schadauer, A.F. Geiss, J. Srajer, B. Siebenhofer, P. Frank, C. Reiner-Rozman, B. Ludwig,  
608 O.M. Richter, C. Nowak, R.L. Naumann, Silica nanoparticles for the oriented encapsulation of  
609 membrane proteins into artificial bilayer lipid membranes, *Langmuir*, 31 (2015) 2511-2516.
- 610 [45] A. Bazzone, W.S. Costa, M. Braner, O. Calinescu, L. Hatahet, K. Fendler, Introduction to  
611 solid supported membrane based electrophysiology, *J. Vis. Exp.*, (2013) e50230.
- 612 [46] P. Schulz, B. Dueck, A. Mourot, L. Hatahet, K. Fendler, Measuring ion channels on solid  
613 supported membranes, *Biophys. J.*, 97 (2009) 388-396.
- 614 [47] G.R. Heath, M.Q. Li, H.L. Rong, V. Radu, S. Frielingsdorf, O. Lenz, J.N. Butt, L.J.C. Jeuken,  
615 Multilayered Lipid Membrane Stacks for Biocatalysis Using Membrane Enzymes, *Adv. Funct.*  
616 *Mater.*, 27 (2017) Artn 1606265.
- 617 [48] L.J.C. Jeuken, S.D. Connell, P.J.F. Henderson, R.B. Gennis, S.D. Evans, R.J. Bushby, Redox  
618 enzymes in tethered membranes, *J. Am. Chem. Soc.*, 128 (2006) 1711-1716.
- 619 [49] M. Li, S.K. Jorgensen, D.G.G. McMillan, L. Krzemniski, N.N. Daskalakis, R.H. Partanen,  
620 M. Tutkus, R. Tuma, D. Stamou, N.S. Hatzakis, L.J.C. Jeuken, Single enzyme experiments reveal  
621 a long-lifetime proton leak state in a heme-copper oxidase, *J. Am. Chem. Soc.*, 137 (2015) 16055-  
622 16063.
- 623 [50] V. Radu, S. Frielingsdorf, S.D. Evans, O. Lenz, L.J.C. Jeuken, Enhanced oxygen-tolerance of  
624 the full heterotrimeric membrane-bound [NiFe]-hydrogenase of *Ralstonia eutropha*, *J. Am. Chem.*  
625 *Soc.*, 136 (2014) 8512-8515.
- 626 [51] S.A. Weiss, R.J. Bushby, S.D. Evans, P.J.F. Henderson, L.J.C. Jeuken, Characterization of  
627 cytochrome *bo*<sub>3</sub> activity in a native-like surface-tethered membrane, *Biochem. J.*, 417 (2009) 555-  
628 560.
- 629 [52] Ó. Gutiérrez-Sanz, P. Natale, I. Márquez, M.C. Marques, S. Zacarias, M. Pita, I.A.C. Pereira,  
630 I. López-Montero, A.L. De Lacey, M. Vélez, H<sub>2</sub>-fueled ATP synthesis on an electrode: mimicking  
631 cellular respiration, *Angew. Chem. Int. Ed.*, 55 (2016) 6216-6220.

- 632 [53] Ó. Gutiérrez-Sanz, C. Tapia, M.C. Marques, S. Zacarias, M. Vélez, I.A.C. Pereira, A.L. De  
633 Lacey, Induction of a proton gradient across a gold-supported biomimetic membrane by  
634 electroenzymatic H<sub>2</sub> oxidation, *Angew. Chem. Int. Ed.*, 54 (2015) 2684-2687.
- 635 [54] A.J. Diaz, F. Albertorio, S. Daniel, P.S. Cremer, Double cushions preserve transmembrane  
636 protein mobility in supported bilayer systems, *Langmuir*, 24 (2008) 6820-6826.
- 637 [55] H. Pace, L. Simonsson Nystrom, A. Gunnarsson, E. Eck, C. Monson, S. Geschwindner, A.  
638 Snijder, F. Hook, Preserved transmembrane protein mobility in polymer-supported lipid bilayers  
639 derived from cell membranes, *Anal. Chem.*, 87 (2015) 9194-9203.
- 640 [56] M.J. Richards, C.Y. Hsia, R.R. Singh, H. Haider, J. Kumpf, T. Kawate, S. Daniel, Membrane  
641 protein mobility and orientation preserved in supported bilayers created directly from cell plasma  
642 membrane blebs, *Langmuir*, 32 (2016) 2963-2974.
- 643